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FOREWORD

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Introduction

Breast cancer predisposition is inherited in an autosomal dominant manner in some individuals (Newman et al 1988). Currently, the inheritance of breast cancer predisposition is clearly associated with a few highly penetrant genes, most notably BRCA1 and BRCA2, in rare families (reviewed in Szabo and King 1995). In our experience with families who exhibit a high risk for inherited predisposition to cancer (families with at least four cases of breast or ovarian cancer), we estimated that approximately 20% are unresolved after screening for mutations in the BRCA1 and BRCA2 genes (Schubert et al 1997). This indicates that other, as yet unidentified, genes exist which are involved in breast cancer predisposition. Some of these currently unidentified genes may convey only a moderately increased risk of breast cancer, potentially with disease expression among mutation carriers dependent on specific environmental exposures. Such variably penetrant genetic predisposition could account for a greater population risk of breast cancer than the relatively rare highly penetrant gene mutations, although it would convey less risk to individual heterozygotes. One gene that has been suggested to play a role in moderately increased risk of breast cancer is the gene mutated in Ataxia-Telangiectasia (ATM). This study sought to clarify the role of ATM in breast cancer predisposition. Specifically, this study asked whether ATM heterozygotes are predisposed to breast cancer.

Ataxia-Telangiectasia (AT) is a recessive genetic disorder (reviewed in Lavin and Shiloh, 1997) characterized by progressive cerebellar ataxia, blood vessel lesions (telangiectasias) and immunodeficiencies. Patients affected with AT are prone to develop lymphoma and leukemia and are extremely sensitive to ionizing radiation. Various regions of ATM have been identified as specific functional domains, including a carboxy-terminal protein kinase domain (Savitsky et al 1995a, Savitsky et al 1995b). The ATM gene product has been shown to play an important role in cellular response to DNA damage, particularly that from ionizing radiation, as a component of a cell-cycle checkpoint pathway (reviewed in Hoekstra 1997). All AT patients identified to date have inherited two germ-line mutations at the ATM locus and most mutations identified truncate the ATM protein. Multiple mutations in ATM have been discovered in AT patients worldwide (The Ataxia-Telangiectasia Mutation Database), with some founder effects of particular mutations in certain populations (Stankovic et al 1998, Telatar et al 1998, Chessa et al 1997, Gilad et al 1996). Most AT patients have the classically identified severe disease phenotype, however some studies have found evidence of particular ATM mutations which are associated with variant phenotypes (Stankovic et al 1998, Gilad et al 1998, Bar-Shira et al 1997, McConville et al 1996).

Epidemiological studies of the families of children suffering from AT have shown an increased incidence of cancer, particularly breast cancer, in the relatives of such patients (Athma et al 1996, Easton 1994, Pippard et al 1988, Swift et al 1987). Family studies have also suggested that exposure to ionizing radiation increases cancer risk in ATM heterozygotes (Swift et al 1991), which is a compelling idea, particularly given the sensitivity of AT patients to radiation and the known function of the ATM gene product in cellular response to DNA damage. The focus of our study was to investigate this question by examining breast cancer patients, both at a population level as well as those who have a phenotype that indicates possible involvement of ATM such as previous ionizing radiation exposure or an extreme response to such radiation, for ATM heterozygosity. Such a breast cancer patient-based approach complements the studies of AT families which have already been reported.

Body of Report

This study was based on the hypothesis that ATM heterozygotes have an increased susceptibility to breast cancer, particularly when exposed over the course of a lifetime to cellular damage such as ionizing radiation. We originally proposed to screen a large, population-based series of breast cancer patients completely for alterations in the ATM gene. However, more recent reports that ATM heterozygosity is not found in a large number of breast cancer patients (Chen et al 1998, FitzGerald et al 1997, Vorechovsky et al 1996a, Vorechovsky et al 1996b) and reports of founder mutations in ATM with differing effects (and therefore potentially differing cancer risks; (Stankovic et al 1998, Telatar et al 1998) lead us to redirect our efforts in a more focused manner than was originally proposed. The studies of Vorechovsky et al, FitzGerald et al and Chen et al examined series of breast cancer patients for ATM gene mutations without finding a significant number of mutations. We feel that these results are important but not definitive; it may be that ATM mutations overall are not common in the general breast cancer population but that some founder mutations are frequent in some populations. Alternatively, it may be that ATM mutations are more prevalent in particular sub-populations of patients who have undergone triggering environmental exposure and who may have distinct cancer phenotypes. Studies indicate the possibility of particular ATM mutations which lead to distinct sub-phenotypes (Gilad et al 1998, Bar-Shira et al 1997, Taylor et al 1997, McConville et al 1996), leaving open the possibility that breast cancer susceptibility is one such sub-phenotype arising from particular ATM mutation(s). Evidence for such breast cancer predisposing ATM alleles is found in the recent report of Stankovic et al (1998), which describes a founder mutation in Great Britain that may lead to a greater susceptibility to breast cancer due to its particular effects on the ATM protein. This British mutation is also interesting as the increased risk of cancer is associated with a less severe AT phenotype, possibly due to the particular functional domain of the gene which is affected. It may be that individuals with particular breast cancer predisposing ATM alleles also have a distinct cancer phenotype, potentially involving environmental triggers to induce disease. We therefore altered the focus of our study to examine patients at a population level for such founder mutations as well as to completely screen the gene in a targeted group of breast cancer patients with specific environmental exposures and/or phenotypes.

Given the information outlined above, which was not available at the time of writing our original proposal, we changed the focus of our ATM screening in breast cancer patients. The population of patients that we screened for ATM mutations reflects a redefinition of our original hypothesis, which is that ATM heterozygotes with breast cancer may have particular cancer predisposing mutations or exhibit a particular cancer phenotype. We therefore grouped our study into two parts; the targeted screening of specific portions of the gene encompassing known founder mutations that could predispose to breast cancer at a significant population level in a population-based set of breast cancer patients and controls, and the complete screening of ATM in a selected group of patients with particular environmental exposures or phenotypes which we believe, based on the known biology of ATM, are the most probable to be associated with ATM mutations.

For the targeted screening of particular mutations in a population-based series of breast cancer patients, we analyzed by single-strand confirmation analysis (SSCA) the 17 regions of ATM with the highest number of founder mutations, based on the Ataxia-Telangiectasia Mutation Database, in 141 patient samples. These targeted regions of ATM encompass 28% of

the coding sequence, but contain 43% of all protein truncating ATM mutations and all of the founder mutations reported to date (see Table 1). The patient samples used in this portion of the study are part of the Carolina Breast Cancer Study (CBCS), a population-based study of both African-American and Caucasian women diagnosed above and below the age of 50 (Newman et al 1995). CBCS samples were sent to us as anonymously coded DNA samples and the University of Washington Institutional Review Board approved our use of them in this study. The CBCS samples are ideal for examining the question of ATM in breast cancer susceptibility as extensive clinical and environmental exposure information, including radiation exposure, is already recorded from each participant. Records from any patient who is found to have a mutation in ATM would therefore be able to be examined for unusual environmental exposures or clinical phenotype. Tumor blocks are also available from most CBCS patients for LOH analysis in any patient who was shown to have an ATM mutation. The series of 141 CBCS breast cancer patients screened for ATM mutations includes 60 African-American and 81 Caucasian women, evenly distributed in age at diagnosis above and below the age of 50. To the best of our knowledge, this series of African-American breast cancer patients is the most extensively studied for ATM mutations to date. In the case of a SSCA variant being detected in the initial patient screen, we also analyzed that segment of ATM in a set of 138 CBCS controls (63 African-American and 85 Caucasian women) which were ascertained to match the patients as closely as possible in age. As not all segments of ATM had positive results in the initial SSCA screen, we analyzed 13 regions of ATM in the controls as well as the patients. Results of the SSCA and variant sequencing are shown in Table 2. Several novel variants were detected as shown in Table 2 but none were clearly associated with breast cancer incidence in this population.

The second group of breast cancer patients included in our ATM analysis is that of patients with a distinct phenotype that we believe indicates possible ATM involvement in their cancer based on the known biology of ATM. These patients were selected from the following groups: previous radiation exposure, radiation sensitivity, families with at least 3 cancer cases and the common inheritance of a single ATM allele between affected members, or a breast cancer patient who has had a child with AT. Samples from patients in this targeted series were initially screened by SSCA in the selected regions outlined above (see Table 1), followed by a complete screen for ATM mutations through the entire coding sequence by the protein truncation assay (PTT; Telatar et al 1998). The PTT assay has the advantage that it is a cDNA based assay that detects splicing variants as well as protein-truncating mutations; it was not possible to do this assay on the CBCS population as no cDNA was available from these patients. More specifically, the patients screened by PTT for mutations in ATM are: 8 breast cancer patients who exhibited a severe sensitivity to radiation therapy for their cancer, 5 families with at least 3 cancer cases and the common inheritance of a single ATM allele between affected family members, 2 patients who had received radiation therapy for Hodgkin Disease before being diagnosed with breast cancer, and one set of parents of an AT child. The 5 families included in this study include 3 families with 3 cases of breast cancer, one family with 2 cases of breast and 2 cases of ovarian cancer, and one family with 1 breast, 2 ovarian, 2 colon and 5 prostate cancer cases. This range of cancer types is consistent with those seen in AT families (Morrell et al 1990). Previous BRCA1 and BRCA2 mutation testing in these families was negative. The mother of the AT child reports that she stood next to her son during his radiation therapy for cancer and that her breast subsequently affected with cancer was within the field of this radiation exposure. Her husband was included

in this series as a positive control for ATM mutation detection, as the father of an AT child he is an obligate heterozygote. In the aggregate, we believe that screening this series of patients had the potential to indicate which phenotype could be associated with ATM mutations and to detect potential individual ATM allele(s) which cause a particular susceptibility to breast cancer. The series of radiation sensitive breast cancer patients is particularly interesting, given previous data regarding the radiation sensitivity of cells taken from AT heterozygote patients (West et al 1995, Thacker 1994) and the role of ATM in cellular response to radiation (reviewed in Hoekstra 1997). Two recent reports have examined similarly radiation-sensitive breast cancer patients for ATM mutations with negative results (Appleby et al 1997, Ramsay et al 1998), however the sum total of patients screened in the two studies was 31, and therefore our 8 patients significantly increase the total number of such patients examined for ATM mutations to date. All patients were enrolled in the study after appropriate informed consent within the structure of our University of Washington Institutional Review Board for Human Subjects agreement.

Since the original submission of this grant, the complete cDNA sequence, genomic organization, and genomic sequence of ATM have been published (Savitsky et al 1995b, Uziel et al 1996, Platzer et al 1997), eliminating the need to obtain this information from other sources. Our preliminary screening strategy for this set of patients was targeted screening by single-strand conformational analysis (SSCA) of genomic DNA for protein truncating ATM mutations with known genomic causes reported multiple times (Table 1). Many of the mutations previously reported in ATM are deletions in cDNA for which the genomic basis is unclear, such variants were disregarded in this targeted screen as they have the potential to be artifacts. The polymerase chain reaction (PCR) primers used in this SSCA analysis were those of Vorechovsky et al (1996a). Fragments screened in this approach include 2581 nucleotides of the 9168 nucleotides of the ATM coding region (Savitsky et al 1995b). This encompasses 43% of the ATM coding region and the adjoining mRNA splicing regions of the exons examined. Results from the screen are shown in Table 2. No variant is clearly associated with breast cancer either in the CBCS cases or in the specific patient subgroups and the only truncating mutation identified in a patient was in the mother of an AT child, an obligate heterozygote.

Table 1: Regions of ATM with Common Protein Truncating Mutations
(The ATM Mutation Database: <http://www.vmmc.org/vmrc/atm.htm>)

<u>Region</u>	<u>Number of individual mutations reported</u>	<u>Total number of mutations reported</u>
exon 12	6	11
exon 15	2	2
exon 16	2	4
exon 20	2	5
exon 24	3	3
exon 39	3	3
intron 40	1	3
exon 43	4	5
exon 46	5	8
exon 51	3	5
exon 52	2	2
exon 53	1	7
exon 54	3	10
exon 55	6	10
exon 58	3	6
exon 63	4	5
exon 64	4	4
17 fragments	54 individual mutations	93 total mutations

The sum total of truncating mutations reported in these genomic regions is 93, or 43% of a total number of 216 truncating mutations reported in the ATM Mutation Database. These regions encompass 2581 bp of the ATM coding sequence, or 28% of the entire 9168 bp.

Table 2: Data from SSCA analysis of selected genomic regions of ATM

A. CBCS Patients

Region	Times the same variant was detected (by race)	Sequencing results ¹
exon 12	8 (6 African-American / 2 Caucasian)	1236-3 T _N polymorphism
exon 12	1 (African-American)	1591 G>C (Ala>Pro) ²
exon 15	1 (African-American)	2096 A>G (Glu>Gly) ²
exon 16	1 (Caucasian)	2250+22 A>C (intronic)
exon 20	1 (Caucasian)	2838+28 ins T (intronic)
exon 39	1 (African-American)	5558 A>T (silent) ²
intron 40	1 (African-American)	gDNA 96757 A>C (intronic)

B. CBCS Controls³

Region	Times the same variant was detected (by race)	Sequencing results ¹
exon 16	1 (Caucasian)	2250+22 A>C (intronic)
exon 39	3 (Caucasian)	5558 A>T (silent) ²
exon 52	1 (Caucasian)	7390 T>C (silent)

C. Non-CBCS patients (selected by phenotype)

Region	Times the same variant was detected (phenotype)	Sequencing results ¹
exon 12	1 (African-American Hodgkin Disease patient)	1591 G>C (Ala>Pro) ²
exon 39	1 (Caucasian family; does not segregate with disease)	5588A>T (silent)
exon 50	1 (Caucasian AT heterozygote)	6996 ins A (truncating)
exon 63	1 (Caucasian family; does not segregate with disease)	9011 A>T (silent) ²

¹ Numbers given are based on the ATM cDNA, nt 1 is the start of translation.

² Previously unreported variant (Ataxia-Telangiectasia Mutation Database)

³ CBCS controls were not analyzed in regions where no variants were detected in patients

Key Research Accomplishments

- A case-control series of both Caucasian and African-American breast cancer patients were analyzed for recurrent mutations in the ATM gene.
- A series of 8 breast cancer patients who exhibited an extreme response to radiation therapy were analysed for mutations in the ATM locus.
- Breast cancer patients from 5 families with at least 3 cases of cancer and the common inheritance of an ATM allele were screened for mutations at ATM.
- 2 patients who developed breast cancer subsequently to treatment for Hodgkin Disease were screened for mutations in the ATM locus.

Reportable Outcomes

The following journal articles were produced during the duration of this grant support:

Schubert EL, Lee MK, Mefford MC, Argonza RH, Morrow JE, Hull J, Dann JL, King M-C (1997) BRCA2 in American families with four or more cases of breast or ovarian cancer: recurrent and novel mutations, variable expression, penetrance, and the possibility of families whose cancer is not attributable to BRCA1 or BRCA2. *Am J Hum Gen* 5:1031-1040.

Schubert EL, Mefford HC, Dann JL, Argonza RH, Hull J, King M-C (1997) BRCA1 and BRCA2 mutations in Ashkenazi Jewish families with breast and ovarian cancer. *Genetic Testing* 1:41-46.

Neuhausen SL, Godwin AK, Gershoni-Baruch R, Schubert EL, Garger J, Stoppa-Lyonnet D, Olah E, Csokay B, Serova O, Lalloo F, Osorio A, Stratton M, Offit K, Boyd J, Caligo MA, Scott RJ, Schofield A, Teugels E, Schwab M, Cannon-Albright L, Bishop T, Easton D, Benitez J, King M-C, Ponder BAJ, Weber B, Devilee P, Borg A, Narod SA, Goldgar D (1998) Haplotype and phenotype analysis of nine recurrent BRCA2 mutations in 111 families: results of an international study. *American Journal of Human Genetics* 62(6):1381-1388.

Welsh PL, Schubert EL, King M-C (1998). Inherited breast cancer: an emerging picture. *Clinical Genetics* 54(6):447-458.

The European Breast Cancer Linkage Consortium (1999) Carrier risks in BRCA2 mutation carriers. *Journal of the National Cancer Institute* 91(15):1310-1316.

Schubert EL, Lee MK, and King, M-C. Single nucleotide polymorphisms in the estrogen receptor locus (ESR1) and inherited breast cancer. In press: *Journal of Steroid Biochemistry and Molecular Biology*.

Schubert EL, Hale KP, Newman B and King, M-C. Molecular genetic analysis of ATM in breast cancer patients and controls. Manuscript in prep.

The following abstracts were presented at scientific meetings during the duration of this grant support:

"Possible Linkage of Breast Cancer to Chromosome 6q25 and Evaluation of Estrogen Receptor as a Candidate Gene" Schubert EL, Walsh TD, Mefford HC, Cloud R, Lee MK and King M-C. The Twelfth Breast Cancer Linkage Consortium Meeting, September 1998.

"Molecular Analysis of ATM in Breast Cancer Patients and Controls" Schubert EL, Hale KP, Newman B and King, M-C. The Eighth International Workshop on Ataxia-Telangiectasia, February 1999.

Elizabeth L. Schubert, Ph.D. was the only person receiving pay from the research effort covered by this report. The training supported by this grant advanced her scientific career and allowed her to progress to a new position in the laboratory of Dr Peggy Porter at the Fred Hutchinson Cancer Research Center, where she continues to focus on breast cancer research.

Conclusions

After significant screening we did not observe the coincidence of any truncating ATM mutation and breast cancer in patients from either the CBCS series or the more focused group. The variants that we have identified are all silent or missense variants, and do not clearly result in loss of function of the ATM protein. No such nontruncating ATM variant was seen significantly more often in breast cancer patients than in controls, or in any of the groups of patients with particular phenotypes.

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